



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(21) International Application Number:</b> PCT/US98/12504 <b>(22) International Filing Date:</b> 16 June 1998 (16.06.98) <b>(30) Priority Data:</b> 60/050,130                      18 June 1997 (18.06.97)                      US <b>(71) Applicant (for all designated States except US):</b> BAYLOR COLLEGE OF MEDICINE [US/US]; Suite 186, One Baylor Plaza, Houston, TX 77030 (US). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> TSAI, Ming-Jer [US/US]; 6014 Charlotte, Houston, TX 77005 (US). TSAI, Sophia, Y. [US/US]; 6014 Charlotte, Houston, TX 77005 (US). QIU, Yuhong [CN/US]; 426 Naomi Way, Neshanic Station, NJ 08853 (US). PEREIRA, Fredrick, A. [CA/US]; 8014 Myrtle Lane, Missouri City, TX 77459 (US). O'MALLEY, Bert, W. [US/US]; 639 Ramblewood, Houston, TX 77079 (US). DEMAYO, Francesco [US/US]; 3624 Merrick, Houston, TX 77025 (US). <b>(74) Agent:</b> PAUL, Thomas, D; Fulbright & Jaworski, L.L.P., Suite 5100, 1301 McKinney, Houston, TX 77010-3095 (US).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.          Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>
<b>(54) Title:</b> COUP-TFII: AN ORPHAN NUCLEAR RECEPTOR REQUIRED FOR ANGIOGENESIS  <b>(57) Abstract</b>  <p>The COUP-TFII gene is essential for angiogenesis. The present invention exploits the role of the COUP-TFII receptor in angiogenesis by the selective use of agonists and antagonists to manipulate angiogenesis. Using agonists of COUP-TFII, angiogenesis can be enhanced, making the agonist a suitable treatment for wounds, promoting recovery from skin-graft surgery and organ transplants. Using COUP-TFII antagonists, angiogenesis can be inhibited, which makes it a suitable treatment for tumor inhibition.</p>		

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**COUP-TFII: AN ORPHAN NUCLEAR RECEPTOR  
REQUIRED FOR ANGIOGENESIS**

**BACKGROUND OF THE INVENTION**

**1. Field of the Invention**

5           The present invention relates to the use of  
agonists and antagonists of the COUP-TFII receptor for  
manipulating angiogenesis, i.e., inhibiting it for  
treatment of tumors, and diabetes complications and  
enhancing it in the case of wound treatment and  
10          congenital heart diseases.

**2. Description of the Related Art**

          The proliferation of new capillary blood vessels  
from a vascular bed is known as angiogenesis. The  
molecular mechanisms of angiogenesis are poorly  
15          understood, which limits any attempt to design a method  
to control angiogenesis at the gene level. Preliminary  
work has been performed to uncover the genetic control  
of angiogenesis. Studies in VEGF and TIE knockout mice  
reveal that each gene plays a key role in angiogenesis.  
20          Risau W., *Mechanisms of angiogenesis*. Nature, 386,  
671-674 (1997). For instance, in TIE-2 (a TIE  
receptor) knockout mice, capillaries do not sprout  
blood vessels in areas such as the perineural plexus.  
Id. A more thorough understanding frustrates an  
25          attempt to design a method to manipulate angiogenesis  
at the gene level, though such a method would of course  
be highly beneficial.

          Most, if not all, solid tumors are capable of  
continuously inducing the proliferation of capillary  
30          blood vessels from the host vascular bed. Folkman J.  
and Haudenschild C., *Angiogenesis in vitro*. Nature,  
288, 551-556 (1980). Moreover, angiogenesis appears to

be important for the continued growth of solid tumors. *Id.* Indeed, extensive vascularization is observed in end-stage tumors. Hanahan D. and Folkman J., *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis*. Cell, 86, 353-364 (1996). Therefore, inhibition of angiogenesis at the tumor site could be an effective means to control tumor growth.

Similarly, effective tissue and organ transplant requires sufficient blood vessel growth in the newly-grafted tissue or organ; hence, promoting angiogenesis would enhance wound healing, including wounds from tissue/organ transplants.

The steroid/thyroid hormone receptor superfamily consists of a group of related ligand-activated transcription factors, which are regulators of gene expression. More than half of the members of the superfamily, which have a putative ligand-binding domain, but for which no ligands have yet been identified, are termed orphan receptors. See, e.g., Tsai M-J. and O'Malley B. W., *Molecular mechanisms of action of steroid/thyroid receptor superfamily members*. Annu. Rev. Biochem., 63, 451-486 (1994).

Among the orphan receptors, the COUP-TFs are the most studied. They have the largest sub-group of receptors, potency to interfere with the functional activities of other members of the superfamily, and exceptional evolutionary conservation with greater than 89% homology from the fruit fly to man.

The entire inner surface of the vascular system is lined by endothelial cells which regulate key vascular functions such as blood-tissue exchange, blood-tissue-barriers, fibrinolysis and coagulation, and activation of the circulating immune system. Until recently, the genetic and molecular mechanisms that control the development of the vascular system had remained a mystery. Currently, several ligands and their membrane receptors have been implicated in the

processes of vasculogenesis and angiogenesis through biochemical, physiological, molecular and transgenic "knockout" analyses. Risau W., *Mechanisms of angiogenesis*. Nature, 386, 671-674 (1997). These reports are exciting because vascular abnormalities in both mice and humans have been defined by receptor-ligand systems on the vascular endothelial cell. Vasculogenesis involves the differentiation of angioblasts from mesoderm. These differentiated endothelial cells then connect into a vascular plexus and organize into blood vessels. Angiogenesis, on the other hand, involves the growth, remodeling and branching of vessels from pre-existing vessels. Recent studies of endothelial growth factors and their receptors have demonstrated that multiple steps are involved in establishing the vascular system. It has been shown that disruption of Flk-1, a vascular endothelial growth factor (VEGF) receptor, results in the absence of blood islands and blood vessel formation in mice, suggesting that Flk-1 is essential for differentiation of endothelial cells. In contrast, Flt-1 mutants, another VEGF-specific receptor, could form endothelial cells, but failed to assemble these cells into proper vascular channels, perhaps due to inappropriate endothelial cell-cell or cell-matrix interactions. Embryos deficient of Tie-1 or Tie-2 receptors, another class of tyrosine receptor kinases, failed to establish intact blood vessels or failed angiogenesis, respectively, resulting in edema and hemorrhage.

Identification of ligand agonists and antagonists for COUP-TFII is a crucial first step to the direct controlled regulation of COUP-TFII regulated functions in angiogenesis. Several molecules have been shown to participate in vascular growth and development. These include VEGFs, FGF-1 and FGF-2, HB-EGF, PDGF-B, TGFb and the angiopoietins. Risau W., *Mechanisms of*

angiogenesis. *Nature*, 386, 671-674 (1997). There is also a growing list of naturally occurring polypeptides which are proteolytic fragments of larger proteins that have potent anti-angiogenic activities. These include  
5 angiostatin, thrombospondin-1, proliferin-related protein (PRP), platelet factor 4 and endostatin. Hanahan D. and Folkman J., *Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis*. *Cell*, 86, 353-364 (1996). The presence  
10 of these inhibitors at physiological levels is sufficient to inhibit neovascularization of tumors and inhibit neovascularization in animal models in the presence of angiogenic inducers like VEGF or FGFs. In addition to growth factors and receptors listed above,  
15 other factors, like components of the extracellular matrix and cell adhesion molecules, have been implicated to be involved in establishment of the vascular system. These factors, which include integrins, HGF and VCAM-1 etc., are also excellent  
20 markers for investigating defects in establishment of the embryonic vasculature.

Although COUP-TFs are well characterized biochemically, the precise physiological roles of COUP-TFs are largely undefined. More specifically, the  
25 physiological role of COUP-TFs, specifically in cell-fate specification and differentiation in development and in the adult, remain largely unknown, prior to this application.

A long felt need and desire in this art has been met by the present invention. The invention comprises:  
30 (1) an animal model for the study of angiogenesis and neovascularization; (2) a method for controlling tumor growth and diabetes complications by inhibiting angiogenesis; and (3) a method for enhancing  
35 angiogenesis to promote wound healing, treat certain cardiovascular disease, and so forth.

The present invention provides numerous advantages

over conventional therapies. Angiogenesis is critical to tumor progression and growth. Tumor growth and metastasis require neovascularization. To ensure tumor growth, the tumor induces factors to recruit blood vessels to invade into the tumor itself (angiogenesis). Cancer researchers are currently designing therapies that destroy tumors by cutting-off its blood supply, oxygen and nutrients critical for tumor maintenance and growth. This is contrary to traditional cancer treatments based on attacking the rebel cancer cells directly, by removing them surgically or attempting to destroy them with radiation or chemotherapy. The current strategies are twofold: inducing blood clots to form in the existing vessels supplying the tumor (anti-vascular); or by preventing the formation or remodeling of the blood supply (anti-angiogenic) to a tumor, thereby starving those cells and killing them. In this sense, regulation of the COUP-TFII gene is perfectly suited for the latter strategy of cancer therapy. A dozen or more peptide drugs which are anti-angiogenic compounds, preventing the de novo development of blood vessels or neovascularization, are at the final stages of clinical trials. These strategies promise to be better suited as cancer therapies because they avoid one of the main handicaps of conventional cancer therapies: the development of drug resistance by the cancer cells, which ultimately leads to treatment failure. While antiangiogenic peptides are effective in inhibiting angiogenesis, antagonists for COUP-TFII are better suited for therapy than current angiogenesis peptide inhibitors because its ligand (i) is a small molecule; (ii) can be easily synthesized; (iii) is easy to administer; and (iv) has no or little potential to be allergic.

In addition, the regulation of COUP-TFII can be exploited in other ways. For instance, surgical transplantation—especially plastic surgery—of tissue to

a host requires that the grafted flap tissue be well vascularized to be maintained. Thus, any molecules capable of promoting neovascularization at the site of transplantation would increase the possibility of the graft to be accepted and maintained. Either systemic or topical application of COUP-TFII ligand agonists which promote neovascularization is highly valuable in these plastic surgery procedures.

#### Summary of the Invention

10           An object of the present invention is to provide a method for preventing tumor growth in a mammal.

          An additional object of the present invention is to provide a method for treating cancer in a mammal.

15           Another object of the present invention is to provide a method for treating diabetes complications in a mammal.

          A further object of the present invention is to provide a method for treating cardiovascular disease in a mammal.

20           An additional object of the present invention is to provide a method for promoting wound healing in a mammal.

          Another object of the present invention is to provide a method for enhancing healing of newly grafted tissues or organs in a mammal.

25           A further object of the present invention is to provide a method for stimulating angiogenesis and neovascularization in a mammal.

30           An additional object of the present invention is to provide an animal model suitable for the study of angiogenesis or neovascularization.

          Another object of the present invention is to provide an animal model suitable for the study of cancer.

35           A still further object of the present invention is to provide a method of preparing an orphan receptor,



COUP-TFII.

Thus, in accomplishing the foregoing objects, there is provided in accordance with one aspect of the present invention a method for preventing tumor growth in a mammal comprising administering to a mammal afflicted with a tumor a therapeutic effective amount of an antagonist of COUP-TFII wherein said antagonist inhibits neovascularization induced by cells in said tumor.

In addition, there is provided in accordance with another aspect of the present invention a method for treating cancer in a mammal comprising administering to a mammal afflicted with cancer a therapeutic effective amount of an antagonist of COUP-TFII, wherein said antagonist induces antiangiogenesis.

In addition, there is provided in accordance with another aspect of the present invention a method for inhibiting angiogenesis and neurovascularization in a mammal comprising administering to said mammal a therapeutic effective amount of an antagonist of COUP-TFII, wherein said antagonist inhibits angiogenesis and neurovascularization.

In addition, there is provided in accordance with another aspect of the present invention a method for treating cardiovascular disease in a mammal comprising administering to a mammal afflicted with cardiovascular disease a therapeutic effective amount of an agonist of COUP-TFII, wherein said agonist induces angiogenesis.

In addition, there is provided in accordance with another aspect of the present invention a method for promoting wound healing in a mammal comprising administering to a mammal afflicted with a wound a therapeutic effective amount of an agonist of COUP-TFII, wherein said agonist induces angiogenesis.

In addition, there is provided in accordance with another aspect of the present invention a method for enhanced healing of newly grafted tissue or organ in a

mammal comprising administering to a mammal having newly grafted tissue or organ a therapeutic effective amount of an agonist of COUP-TFII wherein said agonist promotes neovascularization at the site of said grafted tissue or organ.

In addition, there is provided in accordance with another aspect of the present invention a method for stimulating angiogenesis and neovascularization in a mammal comprising administering to said mammal a therapeutic effective amount of an agonist of COUP-TFII, wherein said agonist stimulates angiogenesis and neovascularization.

In specific embodiments of the present invention the agonist is selected from the group consisting of steroids, retinoids, thyroid hormones, vitamin D, oxysterols, prostaglandins or other lipid-soluble compounds.

In other specific embodiments, the COUP-TFII agonist is administered systemically, topically or directly to the affected region.

In addition, there is provided in accordance with another aspect of the present invention an animal model comprising a mouse in which the genomic DNA for the COUP-TFII gene has been inactivated such that said DNA does not produce a functional protein in said mouse.

In addition, there is provided in accordance with another aspect of the present invention a method for preparing an orphan receptor, COUP-TFII, comprising the steps of introducing into a host cell a DNA molecule capable of directing the expression and secretion of COUP-TFII, and introducing into the host cell a signal sequence capable of directing the secretion of the protein from the host cell; growing the host cell in an appropriate medium; and isolating the protein product of said DNA molecule from the host cell.

Other and further objects and features will be apparent from the following description of the

presently preferred embodiments of the invention, which are given for the purpose of disclosure, when taken in conjunction with the accompanying drawings.

#### Brief Description of the Figures

5            Figures 1A through 1D consists of four sagittal microscopic views (whole-mount X-gal staining) depicting vascular development in mouse embryos wildtype (A, C) and COUP-TFII mutants (B, D).

10           Figures 2A through 2D consists of four sagittal microscopic views (anti-PECAM (CD31)) depicting vascular development in COUP-TFII wildtype (A, C) and COUP-TFII mutants (B, D).

15           Figures 3A through 3F show the different stages of heart development viewed for the left side showing the left ventricle (LV) and inflow track/left atrium (LA). The stages of somite (20s, 22s, 24s, 25s, 26s) and the COUP-TFII status wildtype (+/+), heterozygote (+/-) or COUP-TFII mutant (-/-) are shown on each of the figures.

20           Figure 4 shows the stage of development at E9.75-10 in wildtype embryos (A, +/+). The hindbrain (hb), foregut (fg), and right atrium (ra) are shown.

25           Figure 5 shows the stage of embryo development at E9.75-10 in COUP-TFII mutants (B, -/-) and corresponds to the same view seen in Figure 4. In Figure 5, the arrow shows the lack of expansion of the right atrium in COUP-TFII mutants.

30           Figure 6 shows COUP-TFII expression in the developing retina. The developing retina (R), the optic stalk (O), ocular muscles (M), and lens (L) are shown.

35           The drawings are not necessarily to scale. Certain features of the invention may be exaggerated in scale or shown in schematic form in the interest of clarity and conciseness.

### Detailed Description of the Preferred Embodiments

It is readily apparent to one skilled in the art that various substitutions and modifications may be made to the invention disclosed herein without  
5 departing from the scope and spirit of the invention.

The present application demonstrates that the COUP-TFII gene is an essential gene for mammalian development. In mice, for instance, COUP-TFII mutants can only be recovered prior to 10 days of gestation  
10 indicating post-implantation, pre-natal developmental defects. COUP-TFII mutants have defects in formation of the placenta and brain and die due to an inability to develop the complex network of the vascular blood supply system. Indeed, it has been shown that  
15 COUP-TFII mutants have defects in formation of new capillaries, formation which occurs by sprouting or by splitting from their vessel of origin, or angiogenesis. Therefore, COUP-TFII presents a method for manipulating angiogenesis—i.e., inhibiting it in the case of tumor  
20 inhibition, and inducing it in the case of wound healing. The identification of ligand agonists and antagonists for COUP-TFII allows a direct controlled regulation of COUP-TFII regulated functions in angiogenesis.

25 "Agonist" is a compound which interacts with COUP-TFII to promote a transcriptional response.

"Antagonist" is a compound which interacts with or binds to COUP-TFII and blocks the activity of a receptor agonist.

30 "Orphan receptors" is a designation given to a series of cloned receptors whose primary sequence is closely related to the steroid hormone receptors but for which no ligand has been described.

35 "Steroid/thyroid hormone receptor superfamily" is a classification of a group of proteins, some of which are known steroid receptors whose primary sequence suggests that they are related to each other.

"Transfected/Transfection" is a term describing the process of directly introducing DNA into a mammalian cell.

#### **Therapeutic Effective Amount**

5       As used in the present invention, the compound will be considered a therapeutic effective amount if it decreases, delays or eliminates the onset of a proliferative disease, for example, cancer and neoplastic disease; decreases, delays or eliminates the  
10       onset of a cardiovascular disease or provides relief of or a treatment of or partial benefit in improving the cardiovascular state of the individual; improves the effectiveness of, enhances, increases recovery or any other way improves the growth of a tissue graft or an  
15       organ graft; improves the healing of a wound; is effective in decreasing or eliminating any of the complications of diabetes such as retinopathy, nephropathy or neuropathy. The skilled artisan readily recognizes that in many of these cases the compound may  
20       not provide a cure but may only provide partial benefit. A physiological change having some benefits is considered therapeutically beneficial. Thus, an amount of a compound which provides a physiological change is considered an "effective amount" or a  
25       "therapeutic effective amount".

      A compound or composition is said to be "pharmaceutically acceptable" if its administration can be tolerated by a recipient mammal. Such an agent is said to be administered in a "therapeutic effective  
30       amount" if the amount administered is physiologically significant. An agent is physiologically significant if its presence results in a change in the physiology of a recipient mammal. For example, therapeutic effective means: (i) in the treatment of a  
35       proliferative disease like cancer or neoplastic disease, a compound which inhibits the tumor growth or decreases the size of the tumor; (ii) enhances wound

healing; (iii) increases the chances of success of tissue grafts or organ grafts; (iv) decreases the symptoms of cardiovascular disease or improves cardiac activity or prevents congenital heart disease or heart malformation; and (v) decreases or improves the condition of complications associated with diabetes such as nephropathy or neuropathy.

#### **Dosage and Formulation**

The agonists and antagonists (active ingredients) of this invention can be formulated and administered to inhibit or decrease the symptoms of a variety of disease states (including tumors, neoplasty, cancer, diabetes and cardiovascular diseases or enhance or improve the success of treatments, tissue grafts, organ grafts and wound healing) by any means that produces contact of the active ingredient with the agent's site of action in the body of a mammal. These compounds can be administered by any conventional means available for use in conjunction with pharmaceuticals, either as individual therapeutic active ingredients or in a combination of therapeutic active ingredients. They can be administered alone, but are generally administered with a pharmaceutical carrier selected on the basis of the chosen route of administration and standard pharmaceutical practice.

The dosage administered will be a therapeutic effective amount of active ingredient and will, of course, vary, depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient and its mode and route of administration; age, sex, health and weight of the recipient; nature and extent of symptoms; kind of concurrent treatment, frequency of treatment, and the effect desired. These relationships are generally known in the art for compounds having similar effects and can be readily determined by the skilled artisan.

Dosage (composition) suitable for internal

administration in the treatment of proliferative disease generally contain from about 1 to about 500 mg of active ingredient per unit. In these pharmaceutical compositions, the active ingredient will ordinarily be present in an amount of about 0.05 to 95% by weight based on the total weight of the composition.

The active ingredient can be administered orally in solid dosage forms such as capsules, tablets and powders, or in liquid dosage forms such as elixirs, syrups, emulsions and suspensions. The active ingredient can also be formulated for administration parenterally by injection, rapid infusion, nasopharyngeal absorption or dermoabsorption. The agent may be administered intramuscularly, intravenously, or as a suppository. They can be given in divided doses or in sustained released form. Additionally, gene therapy may be used to target the compound. The skilled artisan can readily recognize that the dosage for this method must be adjusted depending on the efficacy of delivery.

Gelatin capsules contain the active ingredient and powdered carriers such as lactose, sucrose, mannitol, starch, cellulose derivatives, magnesium stearate, stearic acid, and the like. Similar diluents can be used to make compressed tablets. Both tablets and capsules can be manufactured as sustained release products to provide for continuous release of medication over a period of hours. Compressed tablets can be sugar coated or film coated to mask any unpleasant taste and protect the tablet from the atmosphere, or enteric coated for selective disintegration in the gastrointestinal tract.

Liquid dosage forms for oral administration can contain coloring and flavoring to increase patient's acceptance.

In general, water, a suitable oil, saline, aqueous dextrose (glucose), and related sugar solutions and

glycols such as propylene glycol or polyethylene glycols are suitable carriers for parenteral solutions. Solutions for parenteral administration contain preferably a water soluble salt of the active ingredient, suitable stabilizing agents and, if necessary, buffer substances. Antioxidizing agents such as sodium bisulfate, sodium sulfite or ascorbic acid, either alone or combined, are suitable stabilizing agents. Also used are citric acid and its salts, and sodium EDTA. In addition, parenteral solutions can contain preservatives such as benzalkonium chloride, methyl- or propyl-paraben, and chlorobutanol. Suitable pharmaceutical carriers are described in *Remington's Pharmaceutical Sciences*, a standard reference text in this field.

Additionally, standard pharmaceutical methods can be employed to control the duration of action. These are well known in the art and include control-release preparations, and can include appropriate macromolecules, for example: polymers, polyesters, polyaminoacids, polyvinyl, pyrrolidone, ethylenevinylacetate, methyl cellulose, carboxymethyl cellulose or protamine sulfate. The concentration of macromolecules as well as the methods of incorporation can be adjusted in order to control release. Additionally, the agent can be incorporated into particles of polymeric materials such as polyesters, polyaminoacids, hydrogels, poly (lactic acid) or ethylenevinylacetate copolymers. In addition to being incorporated, these agents can also be used to trap the compound in microcapsules.

Useful pharmaceutical dosage forms for administration of the compounds of this invention can be illustrated as follows.

Capsules: Capsules are prepared by filling standard two-piece hard gelatin capsules each with 100 milligram of powdered active ingredient, 175



milligrams of lactose, 24 milligrams of talc and 6 milligrams magnesium stearate.

Soft Gelatin Capsules: A mixture of active ingredient in soybean oil is prepared and injected by means of a positive displacement pump into gelatin to form soft gelatin capsules containing 100 milligrams of the active ingredient. The capsules are then washed and dried.

Tablets: Tablets are prepared by conventional procedures so that the dosage unit is 100 milligrams of active ingredient. 0.2 milligrams of colloidal silicon dioxide, 5 milligrams of magnesium stearate, 275 milligrams of microcrystalline cellulose, 11 milligrams of cornstarch and 98.8 milligrams of lactose. Appropriate coatings may be applied to increase palatability or to delay absorption.

Injectable: A parenteral composition suitable for administration by injection is prepared by stirring 1.5% by weight of active ingredients in 10% by volume propylene glycol and water. The solution is made isotonic with sodium chloride and sterilized.

Suspension: An aqueous suspension is prepared for oral administration so that each 5 millimeters contain 100 milligrams of finely divided active ingredient, 200 milligrams of sodium carboxymethyl cellulose, 5 milligrams of sodium benzoate, 1.0 grams of sorbitol solution U.S.P. and 0.025 millimeters of vanillin.

In gene therapy, it is known that a variety of methods can be used by those skilled in the art. In gene therapy, the compounds can also be targeted to specific locations depending on the method used. Any other procedures for use in targeting of compounds can be used. In addition, the compounds can be applied topically or directly to the location in which the intervention is needed.

The present invention provides a method for preventing tumor growth in a mammal comprising

administering to a mammal afflicted with said tumor a therapeutic effective amount of an antagonist of COUP-TFII wherein said antagonist inhibits neovascularization induced by cells in said tumor.

5       The present invention also provides a method for treating proliferative disease in a mammal comprising administering to a mammal afflicted with said proliferative disease a therapeutic effective amount of an antagonist of COUP-TFII wherein said antagonist induces antiangiogenesis.

10       The present invention also provides a method for treating cardiovascular disease in a mammal comprising administering to a mammal afflicted with cardiovascular disease a therapeutic effective amount of an agonist of COUP-TFII wherein said agonist induces angiogenesis.

15       In specific embodiments the cardiovascular disease treated is congenital heart disease and heart malformation.

20       The present invention also provides a method for promoting wound healing in a mammal comprising administering to a mammal afflicted with a wound a therapeutic effective amount of an agonist of COUP-TFII wherein said agonist induces angiogenesis.

25       In another preferred embodiment, the COUP-TFII agonist is administered systemically. In yet another embodiment, the COUP-TFII agonist antagonist can be administered topically.

30       The present invention also provides a method for enhanced healing of newly grafted tissue or organ in a mammal comprising administering to a mammal having newly grafted tissue or organ a therapeutic effective amount of an agonist of COUP-TFII wherein said agonist promotes neovascularization at the site of said grafted tissue or organ.

35       The present invention also provides a method for stimulating angiogenesis and neovascularization in a mammal comprising administering to said mammal a

therapeutic effective amount of an agonist of COUP-TFII wherein said agonist stimulates angiogenesis and neovascularization.

5 In addition, there is provided in accordance with another aspect of the present invention a method for inhibiting angiogenesis and neurovascularization in a mammal comprising administering to said mammal a therapeutic effective amount of an antagonist of COUP-TFII, wherein said antagonist inhibits angiogenesis and  
10 neurovascularization.

In a particular embodiment of the present invention the treated mammal has complications of diabetes, such as retinopathy, nephropathy and neuropathy.

15 In specific preferred embodiments of the present invention the agonist or antagonist of the above-described methods is selected from the group consisting of steroids, retinoids, thyroid hormones, vitamin D, oxysterols, prostaglandins or other lipid-soluble compound.  
20

In preferred embodiments, the COUP-TFII agonist or antagonist is administered systemically, orally, topically or directly to the region to be treated.

25 The present invention also provides an animal model comprising a mouse in which genomic DNA for the COUP-TFII gene has been inactivated and said DNA does not produce a functional protein in said mouse. Inactivation can include a deletion of at least part of the DNA sequence.

30 The present invention also provides a method for preparing an orphan receptor, COUP-TFII, comprising the steps of introducing into a host cell a DNA molecule capable of directing the expression and secretion of COUP-TFII, and introducing into the host cell a signal  
35 sequence capable of directing the secretion of the protein from the host cell; growing the host cell in an appropriate medium; and isolating the protein product

of said DNA molecule from the host cell.

### Example 1

#### Demonstration of Vascular Defects in COUP-TFII Mutants

The following procedure and explanation illustrate  
5 vascular defects of angiogenesis in COUP-TFII mutants.  
In order to facilitate visualization of vasculature  
development in the yolk sac and the embryo proper, the  
vasculature of COUP-TFII mutants in the Tie-1-LacZ  
heterozygote background were examined by staining for  
10  $\beta$ -galactosidase activity of the LacZ gene. Tie-1 is a  
receptor tyrosine kinase which is specifically  
expressed in endothelial cells which line the interior  
and form the tubular vessels. Therefore, whole-mount  
staining of COUP-TFII mutants in the Tie-1-LacZ  
15 background reveals the general architecture of the  
vasculature during embryonic development. For this  
purpose, mice heterozygous for Tie-1-LacZ(+/-) and  
COUP-TFII(+/-) were intermated to subsequently generate  
Tie-1-LacZ(+/-)COUP-TFII(-/-) embryos as COUP-TFII  
20 mutants and the Tie-1-LacZ(+/-)-COUP-TFII(+/+) embryos  
as controls. Figure 1 shows the vasculature of the  
wildtype and the mutants at 9.5 days of gestation. It  
is clearly evident that large vessels and an extensive  
microcapillary network of branching from the large  
25 vessels are seen in the brain vesicles of controls (+/+  
in Figure 1A). In contrast, there are only large  
vessels, slightly dilated in appearance, and very few  
microcapillaries are apparent in the mutants (-/- in  
Figure 1B). Similarly, the large blood vessels and  
30 microcapillary network are well formed in the region of  
the somatic mesenchyme (spine) of the wildtype control,  
while the large vessels appear dilated but grossly  
normal, and the microcapillary network are effectively  
missing in the COUP-TFII mutants (Figure 1C, and 1D).  
35 The presence of the large blood vessels suggests  
differentiation from angioblast to endothelial cells to

form the primary vascular plexus (vasculogenesis) is largely normal in the COUP-TFII mutants. Yet, the pronounced reduction of the microcapillary network in the brain vesicles and the spine regions suggests that the COUP-TFII mutant embryo is defective in angiogenesis, the forming of new capillaries by sprouting or by splitting from their vessel of origin. This observable defect resembles the mutants of angiopoietin, a ligand of Tie-2, a related member of the Tie-1 receptor tyrosine kinase, which are both required for normal vasculature development.

### Example 2

#### Further Demonstration of Vascular Defects in COUP-TFII Mutants

Although the vasculature of Tie-1-LacZ(+/-) heterozygous mice is phenotypically normal, it is important to ensure that the heterozygous Tie-1-LacZ mice do not contribute to the COUP-TFII mutant defects. Thus, we used whole-mount anti-PECAM immunohistochemical studies to visualize the endothelial cells during vascular development in the COUP-TFII wildtype and mutant background alone to further confirm that the observable phenotypes are indeed due to COUP-TFII deficiency. Again, the microcapillary network is very apparent in the wildtype control animals (Figure 2A and 2C). In contrast, the microcapillary network of the COUP-TFII mutants at the same age are severely reduced in the brain vesicles and regions of the spine (Figure 2B and 2D). Whether the observed reduction of the large vessels is due to a delay of development or a consequence of head defects have yet to be defined. Even the large vessels in the brain vesicles of the mutant (Fig. 2B) are reduced as compared to the controls (Fig. 2A).

Examples 1 and 2 convincingly demonstrate that disruption of COUP-TFII gene expression in mice during early embryonic development affects formation of the

vasculature, particularly in formation of the microcapillary network, due to inappropriate angiogenesis. This vasculature defect compromises the ability of the mutants to survive, resulting in embryonic lethality. In addition, current models for the normal development of blood vessels require the recruitment of mesenchymal cells to developing vessels. This is significant since the lack of expression of COUP-TFII gene in this specific compartment results in vascular defects. Therefore, COUP-TFII is one of the first transcription factor proteins identified which is in some way normally required for the development of blood vessels. Thus, the analysis of the COUP-TFII mutants is a model system for the analysis of the processes involved in angiogenesis.

### Example 3

#### Identification of Ligand Agonists and Antagonists of COUP-TFII

Classical steroid hormone receptors (androgen, estrogen, glucocorticoid and progesterone) have been extensively characterized in terms of their ligands. Numerous ligand agonists and antagonists have been identified to regulate physiological processes as diverse as inflammation to pregnancy to abortion to cancers. As a part of the present invention, ligand agonist(s) and antagonists have been identified for the COUP-TFs, which are very important tools for regulation—to activate or repress—COUP-TF specific target genes. The ligands for members of the superfamily comprising COUP-TFII include steroids, retinoids, thyroid hormones, vitamin D, oxysterols and prostaglandins. It is believed that the ligands for orphan receptors of this family are small hydrophobic or amphipathic molecules which include any lipid-soluble compound. COUP-TFII is a typical member of the superfamily of ligand-activated nuclear transcription factors; it has a typical putative-ligand binding domain

(LBD). This LBD is highly conserved within the superfamily and between different members as diverse as from human to *Drosophila* within the COUP-TF subfamily, from which one can infer the existence of a ligand for the COUP-TF subfamily. The LBD has a pocket structure capable of binding a synthetic molecule as a ligand and be regulated. Thus, a shotgun approach is used to identify the ligand of a given receptor in which an extensive battery of purified compounds known to affect angiogenesis are screened. Conversely, classical biochemical purification and concentration of extracts is used in cotransfection assays.

To identify ligands for this superfamily, concentrated lipid extracts from a variety of tissues is prepared to test its ability to activate the receptor in cotransfection assays. This technique is described in: Heyman R. A., Mangelsdorf D. J., Dyck J. A., Stein R. B., Eichele G., Evans R. M., and Thaller C., *9-cis retinoic acid is a high affinity ligand for the retinoid X receptor*. Cell, 68, 397-406 (1992); Forman B. M., Tontonoz P., Chen J., Brun R. P., Spiegelman B. M., and Evans R. M., *15-Deoxy-delta 12, 14-prostaglandin J2 is a ligand for the adipocyte determination factor PPAR gamma*. Cell, 83, 803-812 (1995); Willy P. J., Umesono K., Ong E. S., Evans R. M., Heyman R. A., and Mangelsdorf D. J., *LXR, a nuclear receptor that defines a distinct retinoid response pathway*. Genes & Development, 9, 1033-1045 (1995). Using this technique, ligands have been identified for the retinoid (RAR and RXR), LXR and peroxisome-proliferator activated receptors (PPAR) which include retinoid and cholesterol metabolites, and prostaglandins which have diverse biological functions including teratogenic and anti-diabetic effects.

**Example 4**  
**Identification of Ligand Agonists**  
**and Antagonists of COUP-TFII**

5 In an initial screen for ligands, a chimeric  
receptor is created in which the ligand-binding domain of  
COUP-TFII is fused to the DNA-binding domain of the yeast  
transcription factor GAL4. This technique is described  
in: Willy P. J., Umesono K., Ong E. S., Evans R. M.,  
Heyman R. A., and Mangelsdorf D. J., *LXR, a nuclear*  
10 *receptor that defines a distinct retinoid response*  
*pathway*. Genes & Development, 9, 1033-1045 (1995). The  
resultant GAL4-COUP-TFII expression plasmid is then  
cotransfected together with a GAL4-responsive luciferase  
reporter plasmid into CV-1 cells and challenged with  
15 concentrates from several tissue sources. A significant  
(greater than twofold) induction of luciferase activity  
indicates the presence of a molecule in the extract  
capable of controlling the GAL4-COUP-TFII chimeric  
protein. The lipid extract activity is then fractionated  
20 on reverse-phase high-pressure liquid chromatography  
(HPLC) and the major activating species identified by gas  
chromatography/mass spectrophotometry (GC/MS).

An alternate approach to identification of COUP-TFII  
ligands is the use of an *in vitro* ligand-binding assay  
25 where the binding of a ligand to a steroid receptor  
induces a well defined and easily detected conformational  
change. This technique is fully described in U.S. Pat.  
Appl. Ser. No. 08/448,270, Tsai et al., *Method of*  
*Identifying Hormone Agonists*, which is incorporated by  
reference into the present application. The  
30 conformational change(s) is located within the  
ligand-binding domain and can be detected by partial  
proteolytic digestion or by a fluorescence change. This  
assay is used to detect binding of ligands to COUP-TFII,  
35 and forms the basis for identifying unknown compounds.  
Then, using COUP-TFII as an affinity matrix to trap the  
ligand, based on its characteristic high-affinity and



specificity of binding, the ligand is further purified and identified by HPLC and GC/MS methods as above. The functional activity of this COUP-TFII ligand is then analyzed in chimeric receptor-reporter cotransfection assays and other angiogenesis-specific assays.

*In vitro* analysis of conformational changes has several advantages over the classical *in vivo* cotransfection assay. It is an *in vitro* assay that directly detects ligand-receptor binding. *In vivo* assays cannot distinguish between ligand-dependent and -independent activation processes. The amount of potential ligand that is required for the *in vitro* assay is low because of the small reaction volume (5  $\mu$ l) in comparison to the volumes of culture media and amounts of ligands required for *in vivo* assays. In addition, if the ligand is a hydrophilic compound, it may not be able to cross the cell membrane and enter the cell without an active transport mechanism. Furthermore, the assayed ligand may be toxic to the growth of cells which preclude it from identification by a cell-based assay system. Finally, because the role of COUP-TF ligands on target gene transcription is not determined by the above method—i.e., whether it represses or activates its target gene. Therefore, at least two assay systems are devised to identify COUP-TFII ligands.

#### Example 5

##### Determining the Role of COUP-TFII

##### Ligands in Angiogenesis

After identification of the ligand agonists and antagonists for COUP-TFII, it is necessary to define the role of COUP-TFII ligands in angiogenesis with an appropriate assay. Since COUP-TFII is a transcription factor found within the cell and not a secreted molecule, the established angiogenesis assays need to be modified. COUP-TFII is over-expressed in mesenchymal cells by the adenovirus-mediated expression system, and the resulting

conditioned medium is then prepared and used to assess the induction of angiogenesis, detected by microcapillary sprouting, utilizing the chick chorioallantoic membrane assay. This assay is further described in: Nguyen M., Shing Y. and Folkman J., *Quantitation of angiogenesis and antiangiogenesis in the chick embryo chorioallantoic membrane*. Microvascular Research, 47, 31-40 (1994). Alternatively, COUP-TFII is transfected into bovine capillary endothelial cells, and proliferation, promoting or inhibiting sprouting, in the presence and absence of the ligands, is assessed. Folkman J. and Haudenschild C., *Angiogenesis in vitro*. Nature, 288, 551-556 (1980). Finally, use of the ligand agonists and antagonist in whole-embryo cultures or directly administered to animals is used to analyze their ability for induction or repression of angiogenesis or neovascularization *in vivo*. From the results of these assays, it is determined that COUP-TFII ligands induce capillary sprouting *in vitro* and *in vivo*. Therefore, these assays establish the functional role of the COUP-TFII ligands in regulating angiogenesis.

#### Example 6

##### Role of COUP-TFII in heart development and heart disease

COUP-TFII mutants have a defect in development of the inflow tract which develops into the left atrium. The endothelium of the trabeculae of the early heart is clearly marked with CD31/PECAM immunostaining at E9.5. Shown in Figs. 3A through 3F are different stages of heart development viewed for the left side showing the left ventricle (LV) and inflow tract/left atrium (LA). Wild type (+/+) and heterozygote (+/-) inflow tracts (Figs. 3A and 3C) are seen to expand to form the left atrium between 20 to 22 somites. At 20 somites, the COUP-TFII mutant (-/-) has a relatively appropriate expansion of the inflow tract as compared to similarly

staged wild type or heterozygotes, however, as development proceeds to the 22 somite stage, the mutant atria fail to continue to expand and all mutants result in a thin endothelial tube (arrows) as seen in the 26 somite mutant (Fig. 3E).

In addition, COUP-TFII embryos that survive to E9.75-10 have a defect in the formation of the right atrium. By this stage, wild type embryos (Fig. 4) have a well developed 4 chambered heart with a substantially enlarged right atrium (ra). In contrast, COUP-TFII mutants (Fig. 5) have a lack of this expansion in the right atrium (arrowhead) leading to a relatively constricted channel towards the sinus venosus. (hb, hindbrain; fg, foregut).

The main bloodstream from the umbilical vein enters the right atrial segment of the heart through the inferior vena cava, flows from the right atrium through the opening in the atrial septum into the left atrium, and then enters the left ventricle, which discharges the blood into the trunk of the aorta. An accessory bloodstream enters the heart from above through the superior vena cava. It crosses under the main bloodstream in the right atrium, passing through into the right ventricle, and leaving the heart via the pulmonary trunk. The accessory bloodstream flows through the ductus arteriosus and rejoins the main bloodstream in the descending aorta. Thus, the right atrium is a major crossroads for the flow of blood at this stage of development. The constriction of the right atrium may contribute to the hemorrhage and lethality of the COUP-TFII mutants as the majority of which succumb at this stage.

Interestingly, COUP-TFII has also been implicated in regulating the pressure overload-induced response in the medium-chain acyl-CoA dehydrogenase (MCAD), the key enzyme which catalyzes a rate-limiting step in the fatty acid  $\beta$ -oxidation (FAO) cycle. Fatty acid oxidation is

the major source of energy in the adult mammalian heart. The chief myocardial energy source switches from glucose and pyruvate in the fetal period to fatty acids after birth. Myocardial energy utilization pathways also undergo alterations during cardiac hypertrophy, indeed, reduced capacity for myocardial FAO is linked to cardiac hypertrophic growth.

Classical steroid hormone receptors (androgen, estrogen, glucocorticoid and progesterone) have been extensively characterized in terms of their ligands. Numerous ligand agonists and antagonists have been identified to regulate physiological processes as diverse as inflammation to pregnancy to abortion to cancers. Ligand agonist(s) and antagonists identified for the COUP-TFs and can be very important tools for the regulation, to activate or repress, COUP-TF specific target genes. The known ligands for members of this superfamily include steroids, retinoids, thyroid hormones, vitamin D, oxysterols and prostaglandins. Generally, the ligands for orphan receptors of this family are small hydrophobic or amphipatic molecules. COUP-TFII is a typical member of the superfamily of ligand-activated nuclear transcription factors, it has a typical putative-ligand binding domain (LBD), this LBD is highly conserved within the superfamily and between different members as diverse as from human to *Drosophila* within the COUP-TF subfamily. This suggests the existence of a ligand for the COUP-TF subfamily. Thus, agonists and antagonists of COUP-TFII function are useful in regulating and treating heart diseases contributed by hypertrophic growth.

#### Example 7

##### Role of COUP-TFII in Diabetic complications leading to retinopathy

As seen in Fig. 6, COUP-TFII is highly expressed in the developing retina (R). Expression of COUP-TFII is

detected in the retina by RNA in situ hybridization. COUP-TFII is also detected in the optic stalk (O) and ocular muscles (M) but not in the lens (L). This expression again occurs at stages when the retina is under going vascularization.

There is a long list of complications due to diabetes including microvascular defects, neuropathy, and retinopathy which can lead to lower limb amputations and blindness. Other complications include nephropathy, karatopathy, cataract formation, uncontrollable infection and atherosclerosis which makes diabetes a major risk factor in cardiovascular disease and stroke. Thus, again the use of agonists of COUP-TFII to promote angiogenesis and neovascularization would help to ameliorate many of these complications due to insulin insufficiency.

All patents and publications mentioned in the specification are indicative of the levels of those skilled in the art to which the invention pertains. All patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art readily appreciates that the invention is well adapted to carry out the objectives and obtain the ends and advantages mentioned as well as those inherent therein. The methods of treating proliferative disease, wound healing, tissue graft, organ graft, cardiovascular disease, diabetes with agonists and antagonists to COUP-TFII, the methods of screening for agonists and antagonists to COUP-TFII, animal models, compounds, pharmaceutical compositions, treatments, methods, procedures and techniques described herein are presently representative of the preferred embodiments and are intended to be exemplary and are not intended as limitations of the scope. Changes therein and other uses will occur those skilled in the art which are encompassed within the spirit of the invention or defined by the

scope of the pending claims.

**WHAT IS CLAIMED IS:**

CLAIMS

1           1.    A method for preventing tumor growth in a mammal  
2           comprising administering to a mammal afflicted with said  
3           tumor a therapeutic effective amount of an antagonist of  
4           COUP-TFII, wherein said antagonist inhibits  
5           neovascularization induced by cells in said tumor.

1           2.    A method for treating proliferative disease in a  
2           mammal comprising administering to a mammal afflicted  
3           with said proliferative disease a therapeutic effective  
4           amount of an antagonist of COUP-TFII, wherein said  
5           antagonist induces antiangiogenesis.

1           3.    A method for treating cardiovascular disease in a  
2           mammal comprising administering to a mammal afflicted  
3           with cardiovascular disease a therapeutic effective  
4           amount of an agonist of COUP-TFII, wherein said agonist  
5           induces angiogenesis.

1           4.    The method of Claim 3 wherein the cardiovascular  
2           disease is a congenital heart disease or heart  
3           malformation.

1           5.    The method of Claim 3 wherein the agonist is  
2           selected from the group consisting of steroids,  
3           retinoids, thyroid hormones, vitamin D, oxysterols,  
4           prostaglandins and other lipid-soluble compounds.

1           6.    A method for promoting wound healing in a mammal  
2           comprising administering to a mammal afflicted with a  
3           wound a therapeutic effective amount of an agonist of  
4           COUP-TFII, wherein said agonist induces angiogenesis.

1           7.    The method of Claim 6 wherein the agonist is  
2           selected from the group consisting of steroids,  
3           retinoids, thyroid hormones, vitamin D, oxysterols,

- 1           prostaglandins and other lipid-soluble compounds.
- 1           8.    The method in Claim 6 wherein the COUP-TFII agonist  
2           is administered systemically.
- 1           9.    The method in Claim 6 wherein the COUP-TFII agonist  
2           is administered topically.
- 1           10.   A method for enhanced healing of newly grafted  
2           tissue or organ in a mammal comprising administering to  
3           a mammal having newly grafted tissue or organ a  
4           therapeutic effective amount of an agonist of COUP-TFII,  
5           wherein said agonist promotes neovascularization at the  
6           site of said grafted tissue or organ.
- 1           11.   The method of Claim 10 wherein the agonist is  
2           selected from the group consisting of steroids,  
3           retinoids, thyroid hormones, vitamin D, oxysterols,  
4           prostaglandins and other lipid-soluble compounds.
- 1           12.   The method in Claim 10 wherein the COUP-TFII agonist  
2           is administered systemically.
- 1           13.   The method in Claim 10 wherein the COUP-TFII agonist  
2           is administered directly to the region of the grafted  
3           tissue or organ.
- 1           14.   A method for stimulating angiogenesis and  
2           neovascularization in a mammal comprising administering  
3           to said mammal a therapeutic effective amount of an  
4           agonist of COUP-TFII, wherein said agonist stimulates  
5           angiogenesis and neovascularization.
- 1           15.   The method of Claim 14 wherein the agonist is  
2           selected from the group consisting of steroids,  
3           retinoids, thyroid hormones, vitamin D, oxysterols,  
4           prostaglandins and other lipid-soluble compounds.



1 16. The method in Claim 14 wherein the COUP-TFII agonist  
2 is administered systemically.

1 17. The method in Claim 14 wherein the COUP-TFII agonist  
2 is administered topically.

1 18. A method for inhibiting angiogenesis and  
2 neurovascularization in a mammal comprising administering  
3 to said mammal a therapeutic effective amount of an  
4 antagonist of COUP-TFII, wherein said antagonist inhibits  
5 angiogenesis and neurovascularization.

1 19. The method of Claim 18 wherein said mammal has  
2 complications of diabetes selected from the group  
3 consisting of retinopathy, nephropathy and neuropathy.

1 20. An animal model comprising a mouse in which the  
2 genomic DNA for the COUP-TFII gene has been inactivated  
3 such that said DNA does not produce a functional protein  
4 in said mouse.

1 21. A method for preparing an orphan receptor, COUP-  
2 TFII, comprising:

3 introducing into a host cell a DNA  
4 molecule capable of directing the expression  
5 and secretion of COUP-TFII, and introducing  
6 into the host cell a signal sequence capable  
7 of directing the secretion of the protein from  
8 the host cell;

9 growing the host cell in an appropriate  
10 medium; and

11 isolating the protein product of said DNA  
12 molecule from the host cell.

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Figure 1A

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Figure 1B

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Figure 1C

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Figure 1D

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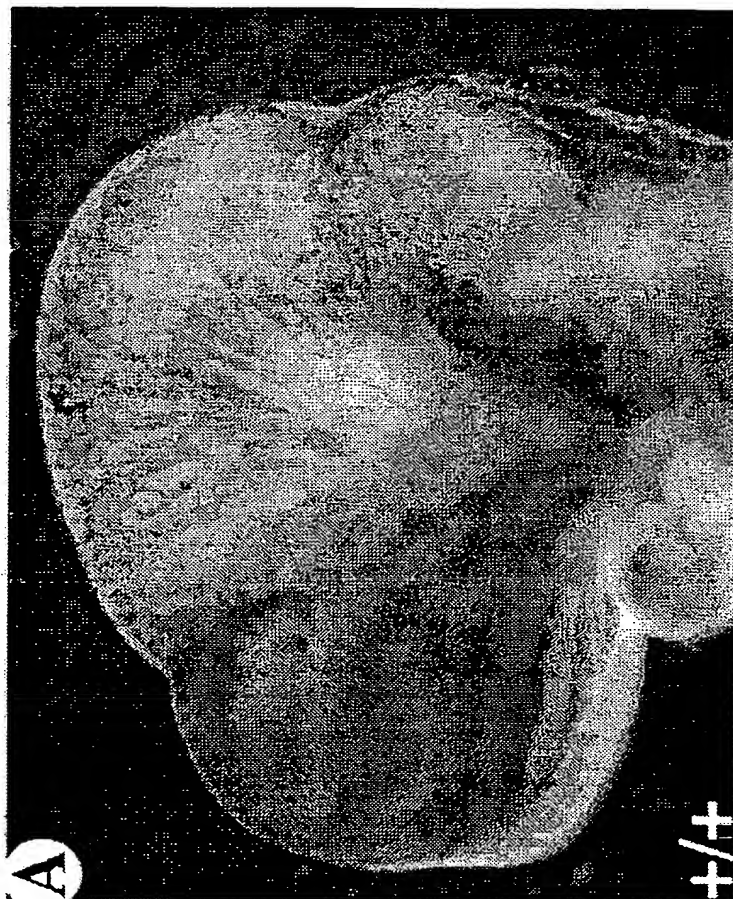


Figure 2A

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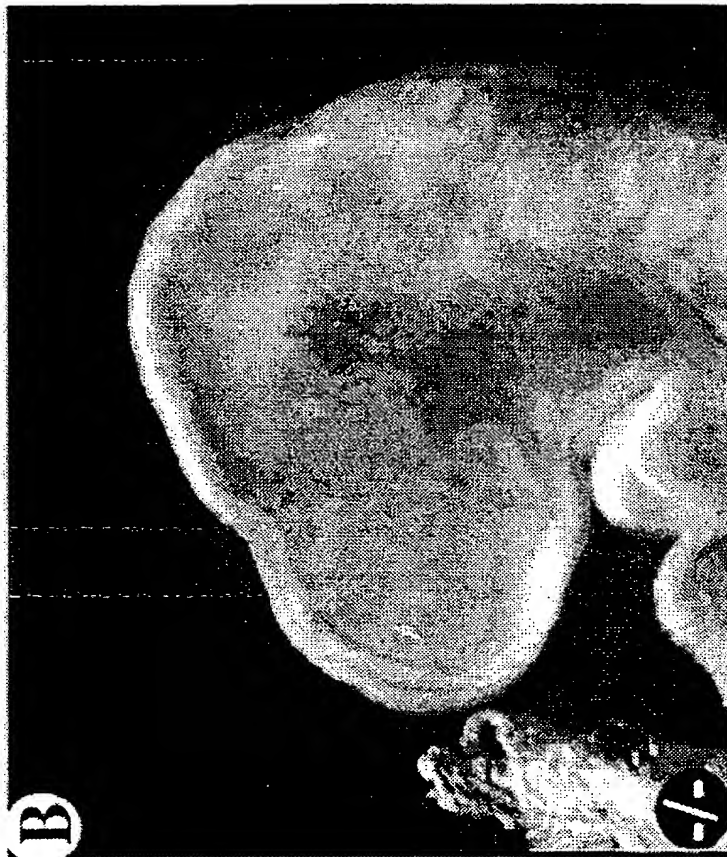


Figure 2B

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Figure 2C

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Figure 2D

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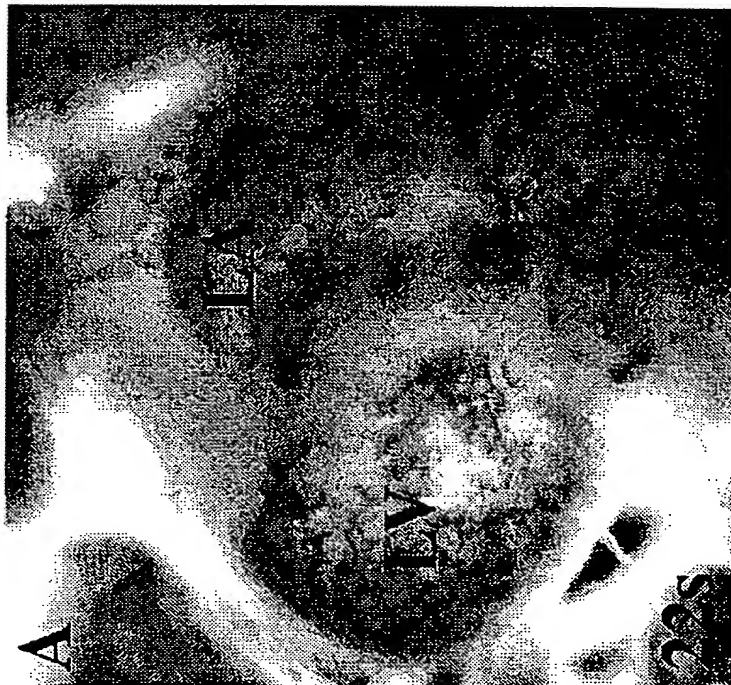


Figure 3A

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Figure 3B

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Figure 3C

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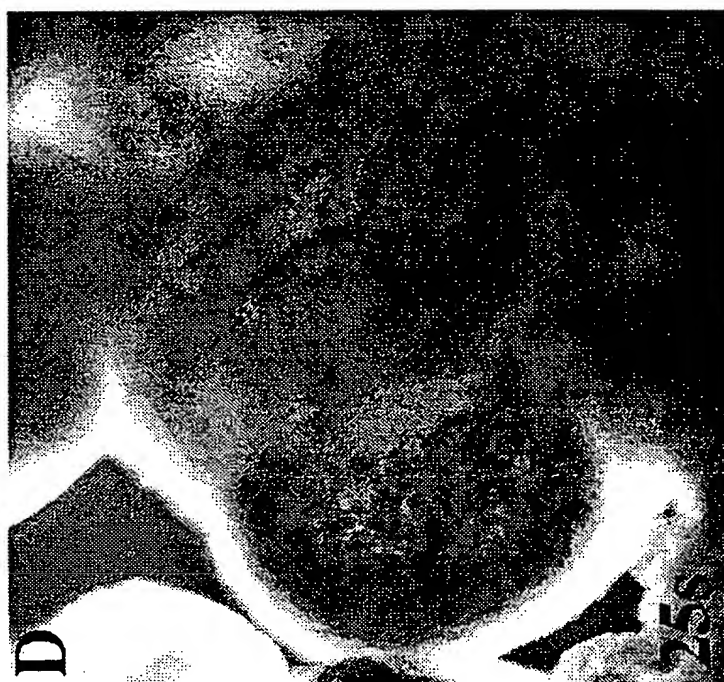


Figure 3D

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Figure 3E

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Figure 3F

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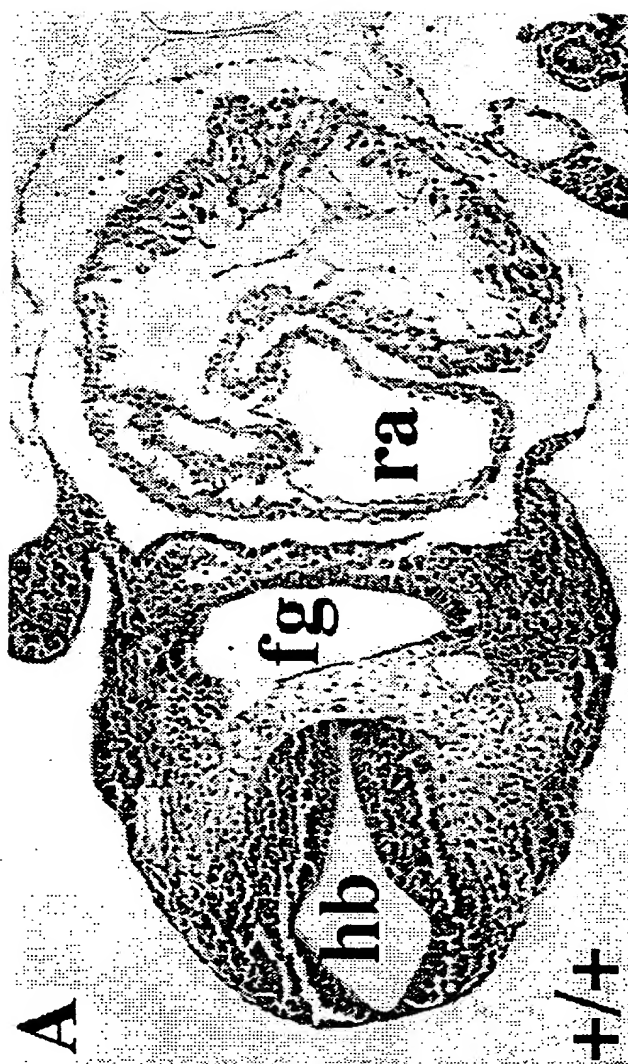


Figure 4

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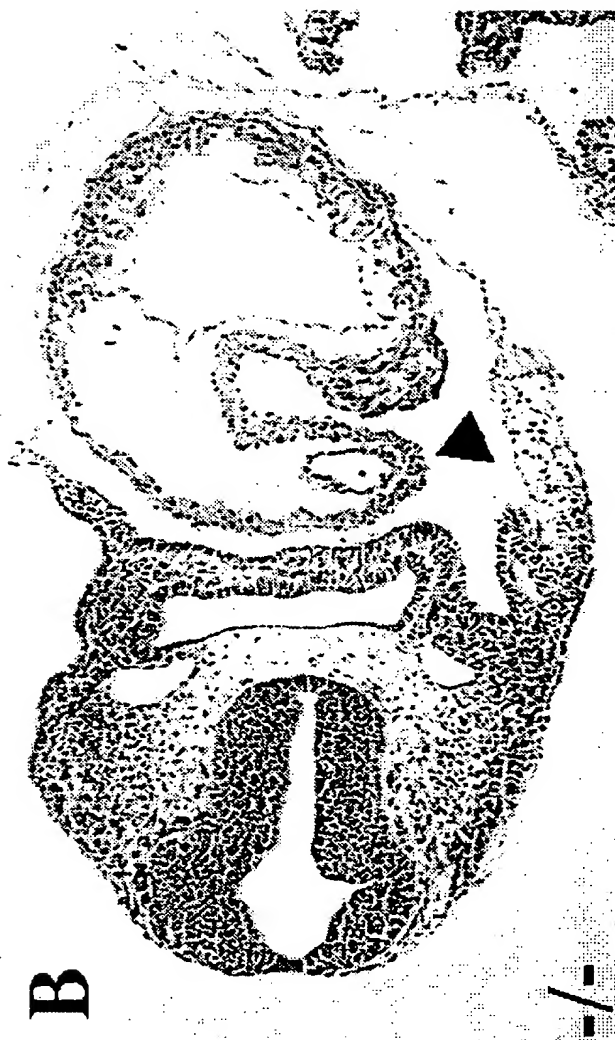


Figure 5

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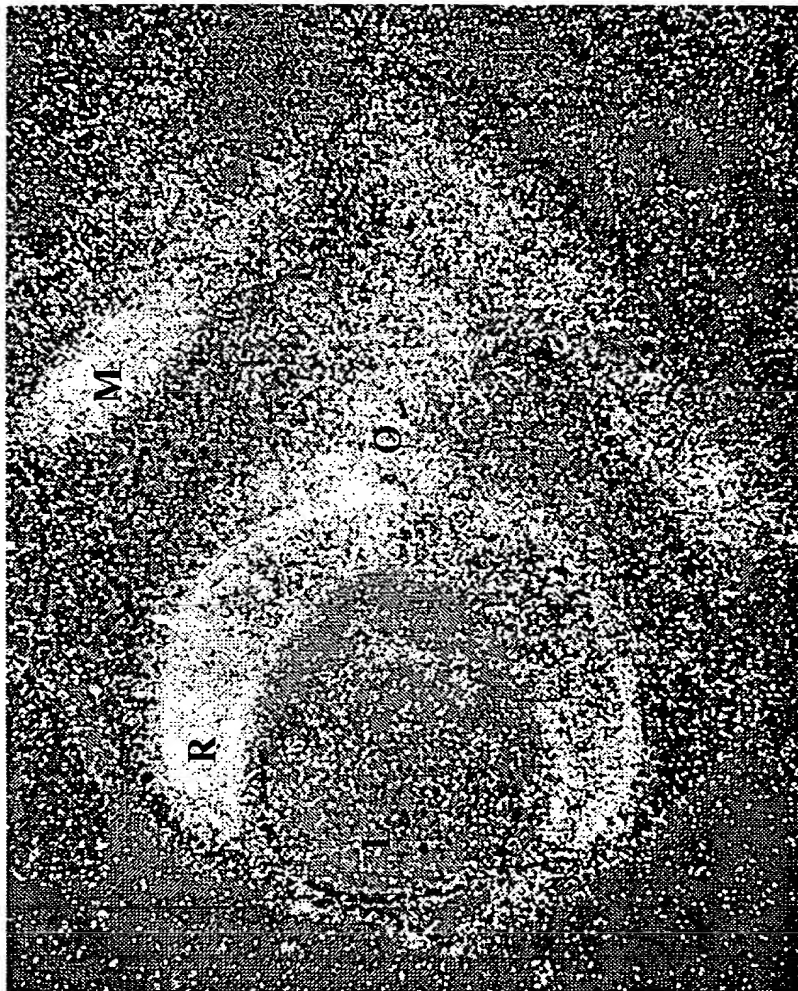


Figure 6

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## INTERNATIONAL SEARCH REPORT

International application No.

PCT/US98/12504

**A. CLASSIFICATION OF SUBJECT MATTER**

IPC(6) : A61K 31/59, 31/56

US CL : 514/167, 177

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 514/167, 177

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched  
NONEElectronic data base consulted during the international search (name of data base and, where practicable, search terms used)  
CAS ONLINE, DERWENT**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 4,897,387 A (IKEKAWA ET AL.) 30 January 1990, see the entire document.	1-21
Y	US 3,442,891 A (BURN ET AL.) 06 May 1969, see the entire document.	1-21

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* *A* *B* *L* *O* *P*	Special categories of cited documents: document defining the general state of the art which is not considered to be of particular relevance earlier document published on or after the international filing date document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) document referring to an oral disclosure, use, exhibition or other means document published prior to the international filing date but later than the priority date claimed	*T* *X* *Y* *Z*	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art document member of the same patent family
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Date of the actual completion of the international search

17 SEPTEMBER 1998

Date of mailing of the international search report

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